THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 15, pp. 12825–12838, April 15, 2011

Escape from p21-mediated Oncogene-induced Senescence Leads to Cell Dedifferentiation and Dependence on Anti-apoptotic Bcl-xL and MCL1 Proteins*

Received for publication, September 17, 2010, and in revised form, February 1, 2011 Published, JBC Papers in Press, February 3, 2011, DOI 10.1074/jbc.M110.186437

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Oncogene-induced senescence (OIS) is a tumor suppressor response that induces permanent cell cycle arrest in response to oncogenic signaling. Through the combined activation of the p53-p21 and p16-Rb suppressor pathways, OIS leads to the transcriptional repression of proliferative genes. Although this protective mechanism has been essentially described in primary cells, we surprisingly observed in this study that the OIS program is conserved in established colorectal cell lines. In response to the RAS oncogene and despite the inactivation of p53 and $p16^{INK4}$, HT29 cells enter senescence, up-regulate $p21^{WAF1}$, and induce senescence-associated heterochromatin foci formation. The same effect was observed in response to B-RAF^{v600E} in LS174T cells. We also observed that $p21^{WAF1}$ prevents the expression of the *CDC25A* and *PLK1* genes to induce cell cycle arrest. Using ChIP and luciferase experiments, we have observed that $p21^{WAF1}$ binds to the *PLK1* promoter to induce its down-regulation during OIS induction. Following 4–5 weeks, several clones were able to resume proliferation and escape this tumor suppressor pathway. Tumor progression was associated with $p21^{WAF1}$ down-regulation and CDC25A and PLK1 reexpression. In addition, OIS and $p21^{WAF1}$ escape was associated with an increase in DNA damage, an induction of the epithelial-mesenchymal transition program, and an increase in the proportion of cells expressing the $CD24^{low}/CD44^{high}$ phenotype. Results also indicate that malignant cells having escaped OIS rely on survival pathways induced by Bcl-xL/MCL1 signaling. In light of these observations, it appears that the transcriptional functions of $p21^{WAF1}$ are active during OIS and that the inactivation of this protein is associated with cell dedifferentiation and enhanced survival.

Oncogene-induced senescence (OIS)[2] is a powerful antitumor mechanism that induces permanent cell cycle arrest in response to abnormal proliferative signals (1). Originally described in cell culture, OIS has been recently shown to occur also *in vivo* as an early protection against carcinogenesis. Its induction involves the combined activities of p53 and $p21^{WAF1}$ to inhibit cell cycle progression and of $p16^{INK4}$ and Rb to induce the transcriptional repression of proliferative genes through heterochromatin formation (2). Senescent cells are characterized by an enlarged morphology, by an increased activity of β-galactosidase, and by the presence of senescence-associated heterochromatic foci (SAHF). Proliferative genes are compacted within these foci to prevent cell cycle progression, generally as a consequence of Rb-mediated silencing. Therefore, through their combined inhibitory effects on cyclin-cdk complexes, the $p16^{INK4}$ and $p21^{WAF1}$ inhibitors play an essential role in OIS induction and in the consequent tumor suppression.

The $p21^{WAF1}$ protein was originally identified as a transcriptional target of the p53 tumor suppressor gene and as an inhibitor of cyclin-cdk complexes and DNA replication (3–6). Gene inactivation studies have also demonstrated essential roles of $p21^{WAF1}$ during DNA damage and chemotherapy responses (7, 8). Besides its classical role as a cyclin-cdk inhibitor, $p21^{WAF1}$ is also recruited to the promoters of cell cycle genes to prevent proliferation. We and others have shown that this protein regulates various transcription factors involved in the G_0/G_1 transition such as NF-κB, Myc, E2F, and STAT3 (9–13). To regulate gene transcription, it is believed that $p21^{WAF1}$ modifies the activity of the CBP histone acetylase and of the cyclin-cdk complexes associated with the initiation complex (14–18).

In light of these observations, we and others have proposed that $p21^{WAF1}$ simultaneously targets growth-promoting genes and cdk activity to induce cell cycle arrest (9, 10, 19). However, although transcriptional functions of $p21^{WAF1}$ have been described following overexpression or in response to chemotherapy treatment, it remains to be determined whether this also occurs during OIS and which promoters are targeted by $p21^{WAF1}$ to restrain an abnormal oncogenic activity. This activity has already been demonstrated for the $p14^{ARF}$ tumor suppressor because this protein can interact with the MYC oncogene to prevent the activation of proliferative genes. Chromatin immunoprecipitation (ChIP) experiments have shown that $p14^{ARF}$ is recruited to the promoter of MYC target genes in association with the MYC-MAX complex (20).

In this study, we have further characterized OIS induction and the corresponding transcriptional functions of $p21^{WAF1}$ in colorectal cells. Using HT29 cells as a model of an established cell line that has inactivated both p53 and $p16^{INK4}$, we surpris-

* This work was supported by a fellowship from Inserm-Pays de Loire (to S. d. C. T.) and by an Equipe Labelisée grant from the Ligue Contre le Cancer (comité du Maine et Loire) and from the Institut du Cancer (to O. C.).

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[2] The abbreviations used are: OIS, oncogene-induced senescence; EMT, epithelial-mesenchymal transition; Rb, retinoblastoma; SAHF, senescence-associated heterochromatic foci; TBS, Tris-buffered saline.

ingly observed that OIS is still functional in these cells, even though this suppressor response is essentially considered to be active in primary cells. The same effect was also noticed in LS174T cells. During OIS, we also found that p21^{WAF1} prevents *CDC25A* and *PLK1* expression and binds to the promoter of the *PLK1* gene. As an essential mechanism of tumor suppression, the OIS pathway has to be inactivated during tumor progression. We effectively observed that several clones escaped this protective pathway and that this was associated with the inhibition of p21^{WAF1} transcription and with the reexpression of *CDC25A* and *PLK1*. In addition, senescence escape was also associated with increased DNA damage and with signs of EMT. Interestingly, these malignant cells show enhanced dependence on Bcl-xL/MCL1 signaling, suggesting that proapoptotic pathways are generated during OIS escape and EMT induction.

Therefore, our results indicate that OIS can be conserved in established cell lines and that p21^{WAF1} functions as a transcriptional inhibitor during this protective pathway. This function is inactivated during tumor escape, and this is associated with genomic instability, cell dedifferentiation, and as a consequence up-regulation of survival signals. These results also suggest that some established cell lines can still be used as an experimental model of OIS response and tumor progression.

MATERIALS AND METHODS

Cell Lines—The human colon adenocarcinoma cell line HT29 and LS174T (American Type Culture Collection, ATCC) were maintained in antibiotic-free RPMI 1640 medium (Lonza). Cultures were supplemented with 10% fetal bovine serum. Cell lines were maintained at 37 °C in 5% carbon dioxide and were tested to rule out mycoplasma contamination.

Cell Transfection and Stable Screening—For transfection experiment, cells were seeded into 60-mm culture dishes and grown until 80% confluence. The empty plasmid pcDN4/T0 and the pcDNA4/H-Ras^{V12} (or B-RAF) were stably co-transfected with the pcDNA6/TR using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instruction. Cells were selected with 100 μg/ml blasticidin (Sigma-Aldrich) and 500 μg/ml zeocin (Invitrogen) for 2 weeks and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum containing 100 μg/ml zeocin and 2.5 μg/ml blasticidin. Oncogene expression was induced by 50 ng/ml doxycycline for 48 h. RasL cell lines were obtained after 5 weeks of H-Ras^{V12} induction. After selection, RasL cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum containing 100 μg/ml zeocin, 2.5 μg/ml blasticidin, and 50 ng/ml doxycycline.

Clonogenic Assay—Cells were seeded at 800 cells into 6-well cell culture plates and incubated at 37 °C in a 5% CO_2 atmosphere. Cells were then treated with 50 ng/ml doxycycline (Sigma-Aldrich) for 8 days, washed twice with PBS, and stained with 0.1% crystal violet. The colonies were then washed twice with water, and colonies exceeding 50 cells were visualized with a Bio-Rad Chemi Doc XRS Imaging device and counted using Quantity One imaging software (Bio-Rad). The survival fraction was determined as the ratio of the number of colonies observed with doxycycline to the number of cells without doxycycline, adjusted to the plating efficiency.

Western Blotting—Following cells lysis at 4 °C (50 mM Tris-HCl, pH 8.1, 1% SDS, 10 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM Na_3VO_4, 50 mM NaF), lysates were sonicated and then boiled for 3 min. Proteins were separated on a SDS-polyacrylamide-containing gel and transferred to a PVDF membrane. Following a 1-h incubation in 3% BSA, Tris-buffered saline (TBS), and 0.1% Tween 20, membranes were incubated overnight at 4 °C with mouse monoclonal anti-H-RAS (1:1000; Santa Cruz sc-29), mouse monoclonal anti-ERK (Tyr^{204}) (1:1000; Santa Cruz sc-7383), goat polyclonal anti-p14^{ARF} (1:600; Santa Cruz sc-8613), rabbit monoclonal anti-p21 antibody (1:1000; Cell Signaling 2947), mouse monoclonal anti-γH2Ax (1:1000; Upstate 05-636), mouse monoclonal anti-α-tubulin (1:1000; Santa Cruz sc5286) HSC70 (1:1000, Santa Cruz sc7298), E-cadherin (1:1000; Abcam 1416). Membranes were then washed twice with TBS with 0.1% Tween 20 and incubated for 1 h with peroxidase-conjugated secondary antibodies (Santa Cruz). Revelation was performed by chemiluminescence with a Bio-Rad Chemi Doc XRS imaging device (Bio-Rad).

Promoter Activity Assay—Cells were seeded into 6-well cell culture plates 24 h prior to transfection. Reporter constructs were transfected into HT29 cells by Lipofectamine (Invitrogen). Twenty-four hours after transfection, luciferase activity was determined by the Dual-Glo luciferase reagent (Promega) and measured using a Packard Topcount scintillation counter. The reporter luciferase was normalized to *Renilla* luciferase, and the ratio of luminescence from the experimental reporter to luminescence from the control reporter was calculated.

Chromatin Immunoprecipitation Assays—ChIP experiments were performed as described previously (21–23). Briefly, HT29 cells were fixed with 1% formaldehyde. After 10 min, cells were washed with ice-cold PBS and lysed with 500 μl of ChIP buffer (50 mM Tris-HCl, pH 8.1, 1% SDS, 10 mM EDTA, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM Na_3VO_4, 50 mM NaF). Chromatin was sheared by sonication to an average size of 500 bp. The chromatin solution was diluted with 1 volume of dilution buffer (2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 1% Triton X-100, 0.1% Nonidet P-40, 150 mM NaCl, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 1 mM Na_3VO_4, 50 mM NaF) and incubated for 1 h at 4 °C on a rotating platform with of protein A-agarose and protein G-Sepharose which was pretreated with sheared DNA salmon sperm. Chromatin was then incubated overnight at 4 °C on a rotating platform with 1 μg of the anti-p21, anti-E2F1, or anti-GAL4 antibody. Following precipitation with protein A-agarose and protein G-Sepharose (pretreated with sheared DNA salmon sperm), chromatin was eluted with elution buffer (1% SDS, 100 mM $NaHCO_3$) for 5 h at 65 °C. DNA was extracted with phenol-chloroform, precipitated with ethanol, allowed to air dry, and then dissolved in 100 μl of sterile H_2O. Four microliters of the DNA samples were then subjected to PCR amplification.

Flow Cytometry—Cells were fixed with 1% formaldehyde at room temperature for 10 min, washed twice with PBS, and incubated with 70% ethanol at −20 °C for 20 min. Cells were then incubated with 1 μg of anti-γH2Ax (1:50; BD Biosciences 560445), anti-CD24 (1:50; BD Biosciences 555427), or anti-

CD44 (1:50; BD Biosciences 555479). Cells were incubated for 1 h at room temperature, and samples were analyzed by FACS (LSR II; BD Biosciences).

siRNA—To down-regulate gene expression, HT29 cells were transfected with 2.5 nM CDKN1A ON-TARGETplus SMARTpool, TP53 ON-TARGETplus SMARTpool, MAPK14 ON-TARGETplus SMARTpool, or ON-TARGETplus Non-Targeting Pool (all from Dharmacon) using DharmaFect-4 (Dharmacon) according to the manufacturer's instructions. H-RAS^{V12} induction was done 24 h after.

Spheroids—10-μl drops containing 1000 cells were suspended on the lids of agar-coated 96-well plates containing 200 μl of culture medium. After a 96-h time period required for cell aggregation, the spheroids were transferred to RMPI 1640 culture medium and maintained for 8 days.

Transwell Migration Assay—The underside of the transwell (8-μm pore size; Falcon) was precoated with Matrigel (100 μg/ml; Sigma E1270) or collagen type I (Col-I; 10 μg/ml). Next, 100 cells were loaded onto the upper chamber, and the lower chamber was filled with serum-free medium. Cells were incubated for 12 h at 37 °C, fixed with 4% paraformaldehyde, and stained with DAPI. Nonmigrating cells retained on the upper side were removed by wiping with a cotton swab. Cells that had migrated through the filter were counted and averaged from five randomly chosen microscopic fields using a 20× objective.

RESULTS

OIS Is Still Functional in HT29 Cells Despite the Inactivation of p53 and p16^{INK4}—Several studies have shown that oncogenes induce growth arrest and senescence, but it is generally believed that this protective pathway occurs essentially in primary cells to restrain the initial events of cell transformation (24, 25). To extend this observation, we wanted to determine whether OIS is conserved in established colorectal cell lines, in the absence of intact p53 and p16^{INK4} signaling. To this end, we generated HT29 cell lines expressing the H-RAS^{V12} oncogene under the control of a doxycycline-inducible promoter. p53 is mutated in this cell line, but p16^{INK4} is not expressed, probably as a consequence of promoter methylation. As expected, Western blot experiments indicated that RAS was up-regulated in response to doxycycline and that the ERK1/2 kinase was activated (Fig. 1*A*). Consequently, we observed under this condition that p14^{ARF} was also up-regulated in response to the RAS oncogene (Fig. 1*B*). Interestingly, MTT and clonogenic assays showed that RAS up-regulation led to long term cell cycle arrest (Fig. 1*C*). In addition, cells exhibited a flat and enlarged morphology illustrative of cells entering the senescence process (data not shown). The induction of the senescence program was also detected by β-galactosidase staining (Fig. 1*D*), a specific marker of cells that do not divide or form colonies. Together, these results indicate that HT29 cells have conserved an intact OIS program in response to aberrant RAS signaling, even though these cells are not primary cells and p53 and p16^{INK4} are inactivated.

p21^{WAF1} Is Up-regulated in Colorectal Cell Lines following Oncogenic Induction—We then determined whether the p21^{WAF1} cell cycle inhibitor was still expressed during OIS in HT29 cells. Interestingly, Western blot experiments showed a significant induction of p21^{WAF1} in response to the oncogene (Fig. 2*A*). We and others have previously described that p21^{WAF1} up-regulation leads to the induction of senescence (26, 27). To determine whether p21^{WAF1} was also involved in OIS activation in colorectal cell lines, cells were transfected with a pool of four different siRNAs directed against the cell cycle inhibitor or control sequences. As expected, p21^{WAF1} expression was down-regulated following H-RAS^{V12} induction (Fig. 2*B*, *lanes 1–4*). Using β-galactosidase staining, we noticed that the inhibition of the cell cycle inhibitor significantly prevented OIS induction (Fig. 2*B*, *lanes 5–8*). As stated above, besides β-galactosidase, OIS is also characterized by the formation of the SAHF heterochromatin foci (see Fig. 6 and Ref. 2). Interestingly, we noticed that the down-regulation of p21^{WAF1} also led to a reduction in the number of these SAHF (Fig. 2*B*, *lanes 9–12*).

We then determined whether p21^{WAF1} was regulated at the transcriptional level during OIS induction. Quantitative RT-PCR experiments showed that H-RAS^{V12} induced a significant up-regulation of the p21^{WAF1} mRNA compared with noninduced cells (Fig. 2*C*). To determine whether this effect occurred at the transcriptional level, we monitored the expression of p21^{WAF1} promoter luciferase constructs following RAS up-regulation. As shown Fig. 2*D*, we found that the p21^{WAF1} promoter-driven luciferase activity was significantly up-regulated during OIS and that the −163/+70 region was sufficient to mediate the response to the RAS oncogene. Because p53 mutants have been recently reported to have unexpected functions (28), we have verified by RNA interference that the p21^{WAF1} promoter is not regulated by the mutated form of p53 under our experimental conditions (data not shown). We then examined the recruitment of the RNA polymerase to the proximal region of the promoter using ChIP. Because serine 2 phosphorylation of the C-terminal domain of the RNA polymerase is a hallmark of transcriptional elongation, ChIP experiments were also performed with polyclonal antibodies directed against the phosphorylated form of the polymerase. GAL4 antibodies were used as controls, and ChIP results were quantified by real-time quantitative PCR (Fig. 2*E*). As expected, the RNA polymerase II and its elongating form were recruited to the p21^{WAF1} proximal promoter following H-RAS^{V12} expression. As a control, PCR analysis did not detect any occupancy of the −2760/−2486 region of the p21^{WAF1} gene (data not shown).

These results indicated that p21^{WAF1} can still be up-regulated in the established HT29 cell line in response to the H-RAS^{V12} oncogene and during OIS induction. To extend this observation, we then asked whether this effect was only related to the HT29 cell line or whether it could also be observed in a different condition. To this end, we generated LS174T cells stably expressing the B-RAF^{v600E} oncogene under the control of a doxycycline-inducible promoter. As expected, Western blot experiments indicated that B-RAF was up-regulated in response to doxycycline and that the ERK1/2 kinase was phosphorylated (Fig. 3*A*, *lanes 1* and *2*). Interestingly, we also noticed that the p21^{WAF1} protein was also significantly up-regulated in this condition (Fig. 3*A*, *lanes 1* and *2*, *middle panel*). Quantitative RT-PCR experiments showed that B-RAF^{v600E} induced the up-regulation of the p21^{WAF1} mRNA compared

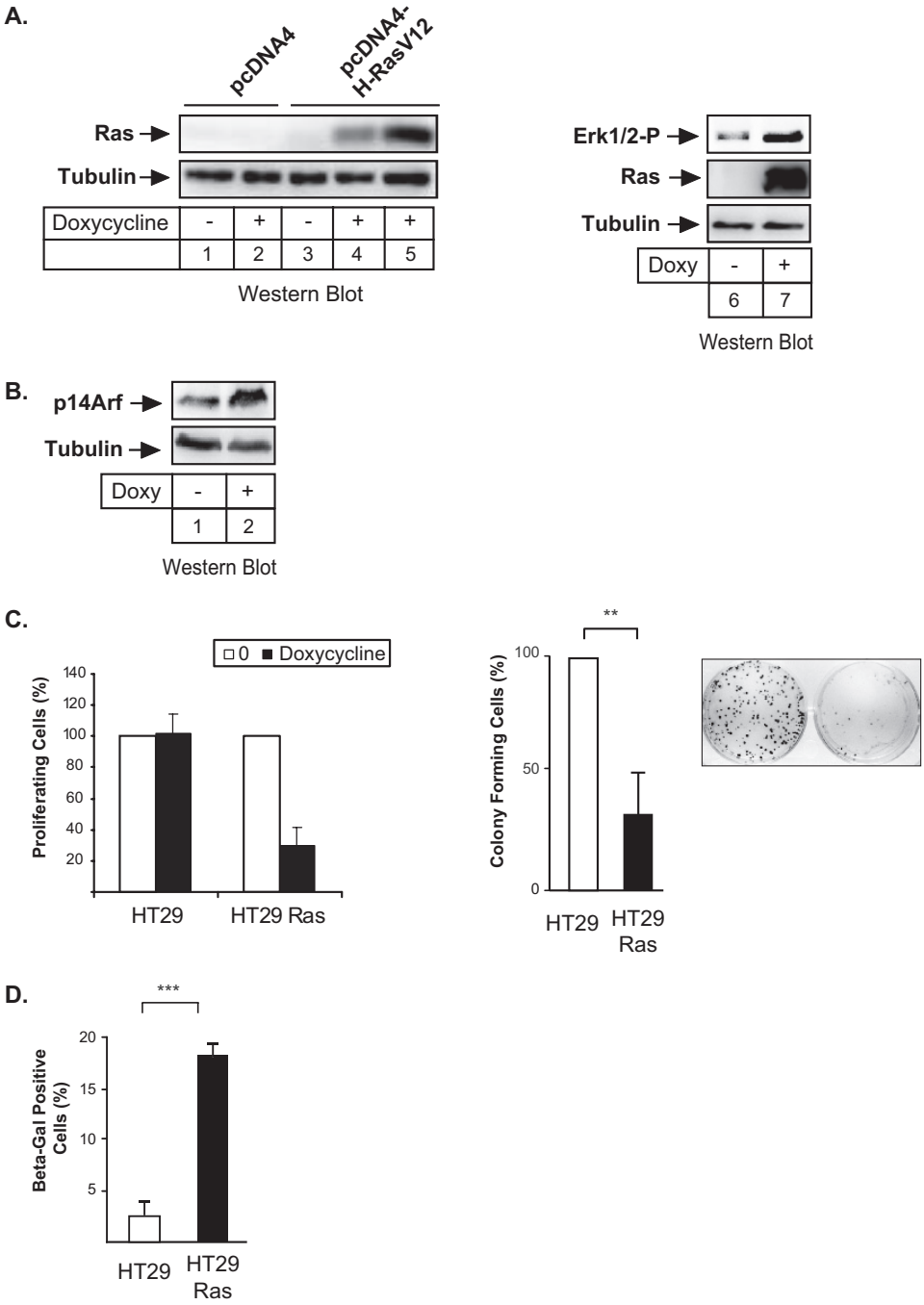


FIGURE 1. **Oncogene-induced senescence is still functional in the established HT29 colorectal cell line.** *A*, to induce H-RAS^{V12} expression, cells were stimulated with 50 ng/ml doxycycline for 24 (*lane 4*) or 48 h (*lanes 2* and *5*), and whole cell extracts were prepared and analyzed with mouse monoclonal anti-H-RAS and mouse monoclonal anti-ERK Tyr^{204}) antibodies as indicated. Tubulin expression was examined as a loading control ($n = 3$). *B*, HT29 cells were stimulated or not with 50 ng/ml doxycycline for 48 h, and whole cell extracts were prepared and analyzed with mouse monoclonal anti-p14^{ARF} antibodies. Tubulin expression was examined as a loading control ($n = 3$). *C*, HT29 cells were plated into 96-well plates, and RAS was induced with 50 ng/ml doxycycline and allowed to grow for 72 h. Cell proliferation was investigated by the MTT assay ($n = 3 \pm$ S.D. (*error bars*), *left*). In parallel, clonogenic assays were performed, cells were incubated with 50 ng/ml doxycycline for 10 days and were stained with crystal violet to count colonies ($n = 5 \pm$ S.D., *right*). *D*, following doxycycline treatment for 7 days, the percentage of SA-β-galactosidase-positive cells was evaluated and is shown as the mean $\pm$ S.D. of at least three independent experiments from a total of at least 300 cells.

with noninduced cells (Fig. 3*A*, *lanes 3* and *4*). Note, however, that this induction was lower than the one observed in HT29 cells in response to H-RAS^{V12}. Compared with HT29 cells, LS174T cells do not express p16^{INK4} but have a functional p53 protein. To determine whether this tumor suppressor was involved in the up-regulation of p21^{WAF1}, its expression was down-regulated by RNA interference before B-RAF^{v600E} induction. Although reduced, the activation of p21^{WAF1} was still detected (Fig. 3*B*, *lanes 1–4*), indicating that the cell cycle inhibitor can be induced in the absence of p53. Interestingly, using clonogenic assays, we also noticed that B-RAF^{v600E} up-regulation led to long term cell cycle arrest in LS174T cells (Fig. 3*C*). Together, these

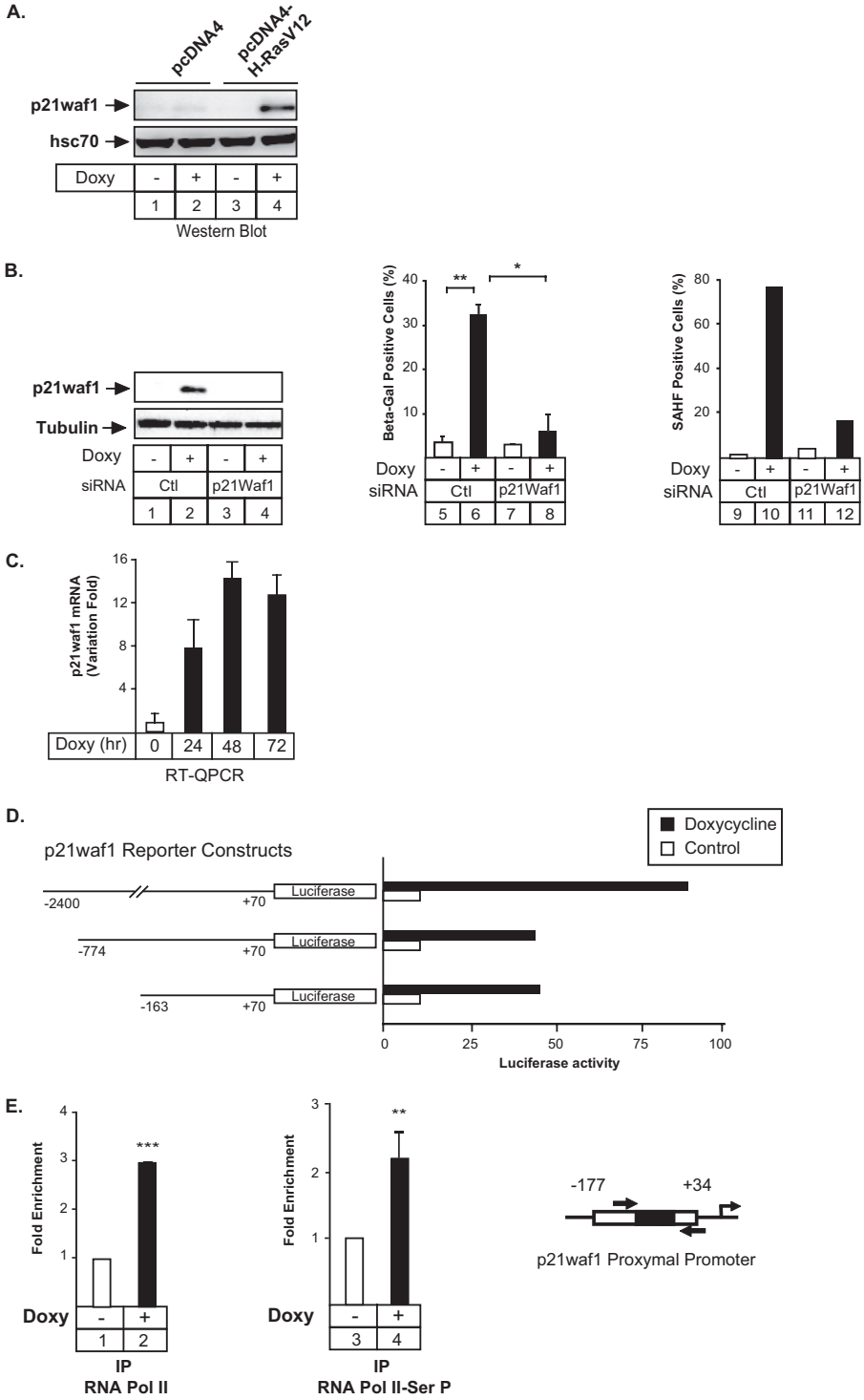


FIGURE 2. **Expression of p21^{WAF1} is induced in response to the RAS oncogene.** *A*, RAS was induced for 48 h, and the expression of p21^{WAF1} was then evaluated by Western blot analysis using total cell extracts ($n = 4$). Hsc70 expression was used as a loading control. *B*, HT29 cells were transfected either with p21-specific siRNA oligonucleotides or control oligonucleotides, and p21^{WAF1} inactivation was then evaluated by Western blot analysis (*lanes 1–4*). Following doxycycline treatment for 7 days, the percentage of SA-β-galactosidase-positive cells was evaluated and is shown as the mean ± S.D. (*error bars*) of at three independent experiments (*lanes 5–8*). In parallel, SAHF formation and chromatin compaction were analyzed following RAS induction in the presence or absence of p21^{WAF1} by immunofluorescence using DAPI staining (*lanes 9–12*, one experiment representative of three). *C*, cells were stimulated as described above for the indicated times, and the amount of the p21^{WAF1} mRNA was determined by quantitative (Q) RT-PCR. Data are expressed as compared with noninduced control value and normalized to RPLPO ($n = 3$, mean ± S.D.). *D*, HT29 cells were transfected with the indicated *p21^{WAF1}* reporter gene and treated or not with doxycycline. Whole extracts were then prepared to measure luciferase activity ($n = 3$). *E*, HT29 cells were treated as described above, and soluble chromatin was prepared from the indicated cells and immunoprecipitated with antibodies directed against the RNA polymerase, its Ser^2 phosphorylated form, or control anti-GAL4 antibodies. DNA was amplified using one pair of primers that covers the p21^{WAF1} proximal promoter. ChIP assays were quantified by real-time PCR compared with GAL4 signal and control region ($n = 3$ ± S.D.).

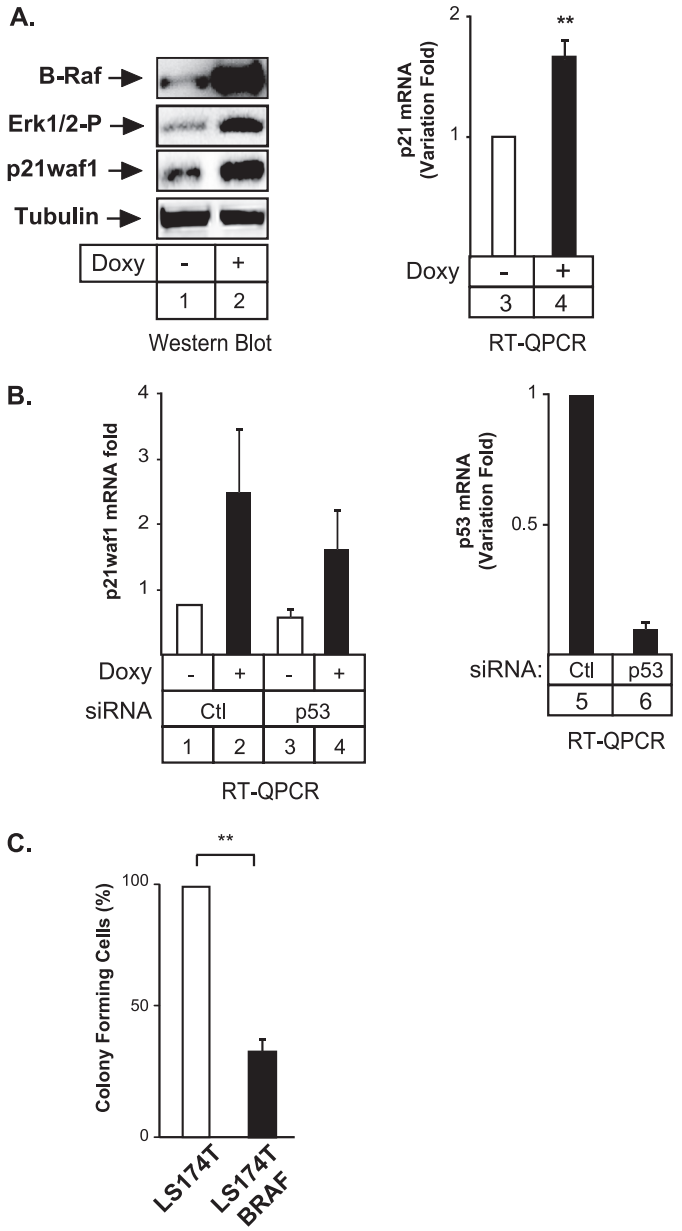


FIGURE 3. **p21^{WAF1} and cell cycle arrest are also induced in response to the RAF oncogene in LS174T cells.** *A*, to induce B-RAF^{V600E} expression, LS174T cells were stimulated with 50 ng/ml doxycycline for 48 h, and whole cell extracts were prepared and analyzed with mouse monoclonal anti-B-RAF and mouse monoclonal anti-ERK (Tyr^{204}) antibodies as indicated (*lanes 1* and *2*). Tubulin expression was examined as a loading control ($n = 3$). In parallel, p21^{WAF1} expression was evaluated by Western blot analysis (*lanes 1* and *2*) or quantitative (Q) RT-PCR (*lanes 3* and *4*). *B*, LS174T cells were either transfected with p53-specific siRNA oligonucleotides or control oligonucleotides, B-RAF was induced for 48 h with 50 ng/ml doxycycline, and p21^{WAF1} expression was then evaluated by RT-QPCR (*lanes 1–4*). The inactivation of the p53 mRNA is presented in *lanes 5* and *6* ($n = 2 \pm$ S.D. (*error bars*)). *C*, LS174T cells were plated into 96-well plates, and B-RAF was induced with 50 ng/ml doxycycline. Clonogenic assays were performed for 7 days, and cells were stained with crystal violet to count colonies ($n = 3 \pm$ S.D.).

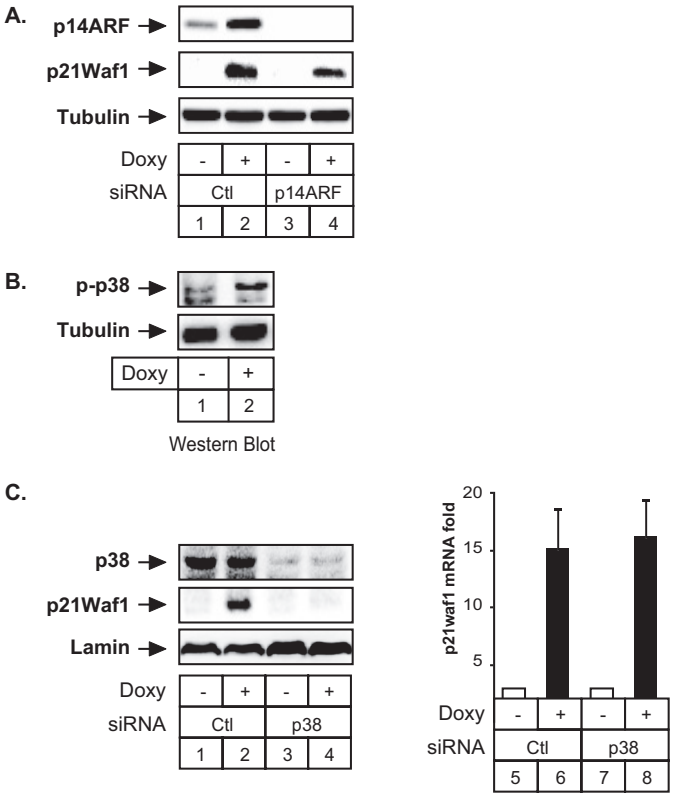


FIGURE 4. **Expression of the p21^{WAF1} protein is regulated by p38 in response to the RAS oncogene.** *A*, HT29 cells were either transfected with p14^{ARF}-specific siRNA oligonucleotides or control oligonucleotides, RAS was induced for 48 h with 50 ng/ml doxycycline, and p14^{ARF} and p21^{WAF1} expressions were then evaluated by Western blot analysis as indicated ($n = 3$). *B*, RAS was induced for 48 h, and the phosphorylation of the p38 kinase was then evaluated by Western blot analysis using total cell extracts ($n = 3$). Tubulin expression was used as a loading control. *C*, HT29 cells were either transfected with p38-specific siRNA oligonucleotides or control oligonucleotides, RAS was induced for 48 h with 50 ng/ml doxycycline, and p38 and p21^{WAF1} expressions were then evaluated by Western blotting or quantitative (Q) RT-PCR analysis as indicated ($n = 3 \pm$ S.D. (*error bars*)).

results indicate that HT29 and LST174T colorectal cells have conserved the ability to up-regulate p21^{WAF1} and induce an OIS program in response to aberrant RAS or RAF signaling.

Expression of the p21^{WAF1} Protein Is Regulated by p38 Kinase—Results presented Fig. 1*B* indicated that the p14^{ARF} tumor suppressor was activated in HT29 cells in response to H-RAS^{V12}. Because this protein is well known to play an important role during senescence (29), we inactivated its expression by RNA interference to determine its effect on p21^{WAF1} regulation. Western blot experiments confirmed as expected that p14^{ARF} was down-regulated following siRNAs transfection (Fig. 4*A*, *lanes 1–4*, *top panel*). However, we did not observe any effect on the expression of p21^{WAF1}, suggesting that p14^{ARF} was not implicated in the up-regulation of the cell cycle inhibitor (Fig. 4*A*, *lanes 1–4*, *middle panel*).

Elegant results have recently shown that the p38 kinase is involved in the regulation of p21^{WAF1} expression and in senescence induction in response to abnormal oncogenic expression (30, 31). We therefore determined the role of this kinase in p21^{WAF1} regulation in our experimental conditions. Results presented in Fig. 4*B*, *lanes 1* and *2*, confirmed that p38 was phosphorylated following RAS induction (note that the antibodies used in these experiments are directed against the three isoforms of p38). To determine whether this kinase was involved in p21^{WAF1} up-regulation, cells were transfected with a pool of four specific siRNAs directed against p38 or with control sequences. As expected, p38 expression was down-regulated (Fig. 4*C*, *lanes 1–4*, *top panel*). Interestingly, the induction

of p21^{WAF1} was not observed in the absence of the kinase (Fig. 4*C*, compare *lanes 1–2* and *3–4*, *middle panel*). By contrast, we did not observe any effect on the mRNA expression of the cell cycle inhibitor, suggesting that p38 regulates the synthesis or stability of p21^{WAF1} as reported previously (30, 31) but not the expression of the gene (Fig. 4*B*, *lanes 5–8*). Together, we concluded from these results that the p21^{WAF1} protein is regulated both at the transcriptional and post-transcriptional levels following RAS induction and OIS activation.

p21^{WAF1} Functions as a Transcriptional Regulator of the PLK1 Gene during OIS—We then determined the effect of OIS on cell cycle progression. Flow cytometry analysis indicated that the percentage of cells in the G_1 phase was not significantly modified whereas an increased number of cells in the G_2 phase was detected after RAS induction, probably due to a reduction of cells in S phase. This distribution did not vary significantly during the 4 days that followed RAS induction (Fig. 5*A*). The expression of the Myc, CDC25A and PLK1 mRNAs was then evaluated because these genes are involved in G_1 and G_2 progression. Results presented Fig. 5*B*, *lanes 3–6*, indicate that OIS induced a down-regulation of the CDC25A and PLK1 mRNAs. Because these two genes are well known to be necessary at the G_1/S and G_2/M transitions, this result is consistent with the flow cytometry results. By contrast, we did not observe any modification of Myc mRNA expression (Fig. 5*B*, *lanes 1* and *2*).

Following chemotherapy treatment, we have already shown that the cell cycle inhibitor binds to cell cycle genes to prevent their expression (13). These observations suggested to us that during OIS, p21^{WAF1} might induce the transcriptional down-regulation of the *CDC25A* and *PLK1* genes through promoter binding. To verify this hypothesis, cells were first transfected with a reporter construct containing the *PLK1* and *CDC25A* promoters (32), in the presence or absence of a p21^{WAF1} expressing vector. Interestingly, a significant down-regulation of reporter gene activity was noticed in the presence of p21^{WAF1} (Fig. 5*C*). Note that it has already been proposed that p21^{WAF1} affects *PLK1* transcription in a different experimental system (27). To confirm this observation, cells were transfected with a pool of four siRNAs directed against p21^{WAF1} or the corresponding control siRNAs, and the expression of PLK1 and CDC25A was then investigated by quantitative RT-PCR analysis. As expected, p21^{WAF1} expression was down-regulated (Fig. 5*D*, *right panel*). Interestingly, the RAS-mediated inhibition of CDC25A and PLK1 was significantly reduced in the absence of the cell cycle inhibitor whereas control siRNA had no significant effects (Fig. 5*D*, compare *lanes 1–2* and *3–4*, *lanes 5–6* and *7–8*). To extend these observations, ChIP experiments were then performed to determine whether p21^{WAF1} binds to the *CDC25A* and *PLK1* promoters in response to abnormal oncogenic signaling. Although we were not able to detect the cell cycle inhibitor on the *CDC25A* proximal promoter, ChIP experiments indicated that p21^{WAF1} was recruited to the *PLK1* promoter following RAS induction (Fig. 5*E*, *lanes 1* and *2*). As a control, PCR analysis did not detect any occupancy of a 5′ control region of the *PLK1* promoter. Although p21^{WAF1} was not found associated with the proximal promoter, the E2F1 transcription factor was detected, and its DNA binding was reduced in response to RAS induction (Fig. 5*E*, *lanes 5* and *6*). Interestingly, E2F proteins have already been described as regulators of the *PLK1* gene (33), and in addition, p21^{WAF1} can associate with E2F1 to prevent its activity (34). Together, these results indicate that p21^{WAF1} binds to the PLK1 gene during OIS to prevent its expression.

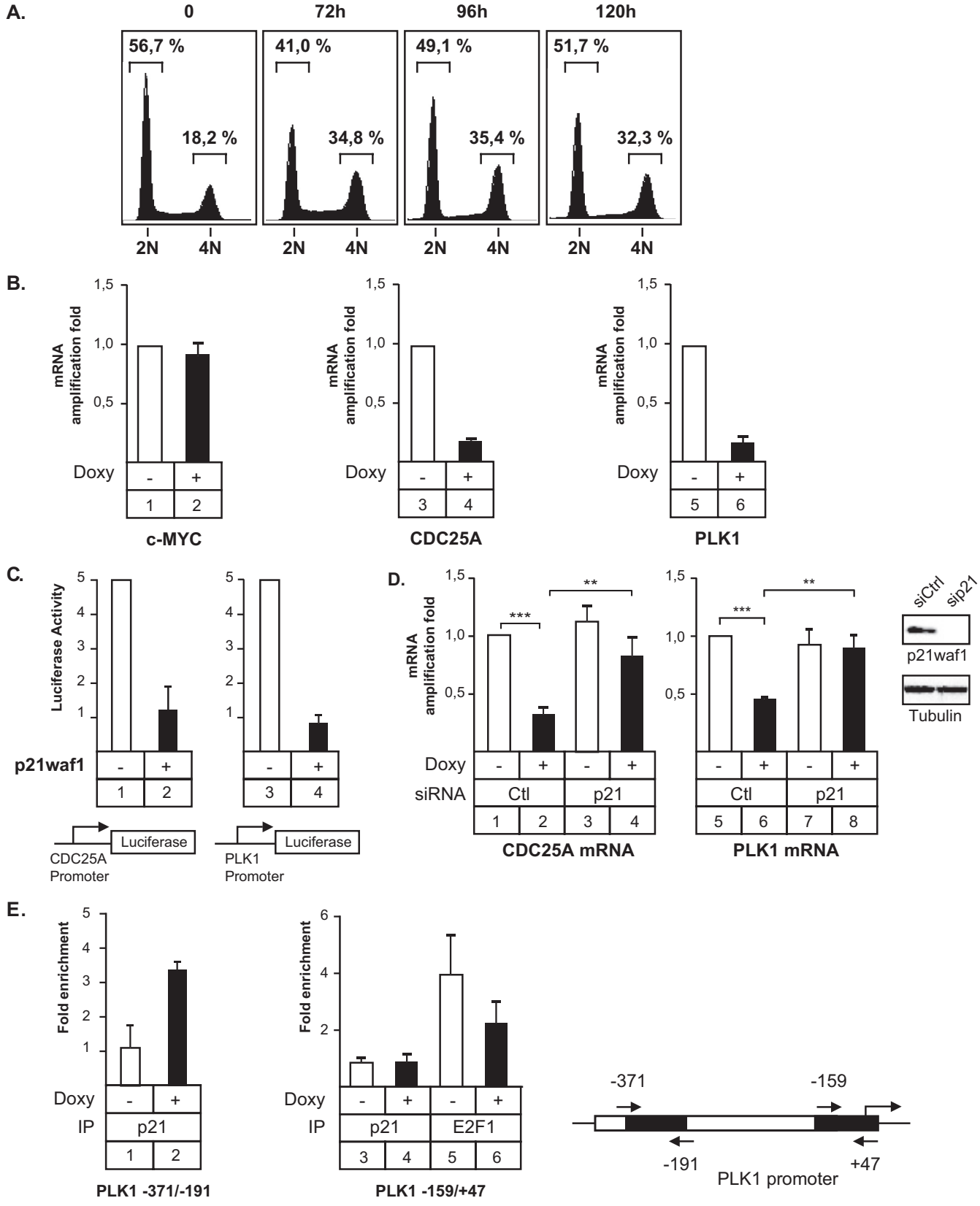
p21^{WAF1} Pathway Is Inactivated following OIS Escape to Allow PLK1 Reexpression—During the course of these experiments, we have noticed that a few emergent clones bypass the OIS pathway (see the experimental protocol in Fig. 6*A*; emergent clones are noted RasL. Note that escape occurred for both HT29 and LST174T cells but that we are presenting results only for HT29 cells). After approximately 4–5 weeks, several clones resumed proliferation while still expressing active forms of RAS and ERK (Fig. 6*B*). This result suggested to us that these cells have inactivated the p21^{WAF1} protective cascade. To verify this hypothesis, Western blotting experiments were performed in several emergent clones to characterize the expression of p21^{WAF1}. As expected, p21^{WAF1} was expressed following RAS induction in parental cells. Interestingly, this up-regulation was lost following OIS inactivation (Fig. 6*C*, *lanes 1–5*). In addition, we also noticed that the expression of the p21^{WAF1} mRNA was down-regulated in the emergent clones (Fig. 6*C*, *lanes 6–9*). Interestingly, p21^{WAF1} inhibition was correlated with a reexpression of the CDC25A and PLK1 mRNAs in the cells that have escaped the OIS arrest (Fig. 6*D*). Therefore, OIS bypass is associated with a down-regulation of p21^{WAF1} and with a reexpression of the CDC25A and PLK1 mRNAs.

p21^{WAF1} Down-regulation and OIS Escape Are Associated with Enhanced Genomic Instability and SAHF Inactivation—DNA damage due to abnormal replication has been recently proposed to function as an anticancer barrier (35, 36). Because p21^{WAF1} plays an important role in response to genomic instability, we then determined whether this response could be observed in our experimental conditions. To this end, HT29 cells were stained with antibodies recognizing the phosphorylated form of histone H2Ax, a marker of DNA double strand breaks. As expected, Western blotting results showed that the induction of the H-RAS^{V12} oncogene led to DNA damage (Fig. 7*A*, *lanes 1* and *2*), probably as a consequence of replicative stress. We then determined whether DNA damage was maintained following OIS escape and p21^{WAF1} inactivation. Interestingly, a significant phosphorylation of histone H2Ax was still detected in the emergent clones (Fig. 7*A*, *lanes 3–7*), indicating that these clones continue to proliferate despite a significant amount of DNA damage. The same effect was noticed when DNA damage was analyzed by FACS (Fig. 7*B*).

As stated above, OIS is also characterized by the formation of SAHF (2). DAPI staining effectively showed an increase in the presence of punctuate heterochromatin foci in the nucleus of cells following RAS induction. Interestingly, this was not observed any more in cells that had escaped the OIS protective mechanism (Fig. 7*C*). Together, these results indicate that OIS bypass and p21^{WAF1} down-regulation are associated in HT29 cells with enhanced genomic instability and SAHF inactivation.

EMT Is Induced in HT29 Cells during p21/OIS Escape—Using breast cancer lines, it has been proposed recently that during senescence inactivation, the down-regulation of p21^{WAF1} is associated with an induction of the EMT and that this leads to

dedifferentiation (37). We then determined whether this was also occurring in the HT29 established cell line. Using a tumor spheroid model, we first observed that parental HT29 cells formed well differentiated round spheroids with strong cell-cell contacts (Fig. 8*A*). As expected, spheroids did not grow following H-RAS^{V12} induction as a consequence of cell cycle arrest.



Interestingly, cells that have inactivated p21^{WAF1} and escaped OIS-generated irregular spheroids. Cells frequently left the spheroid structures, and migration of chains of cells was observed in the surrounding matrix (Fig. 8*A*). The same observation was made using a classical wound-healing test, showing enhanced migration and reduced adhesion of cells that have escaped the p21/OIS pathway (Fig. 8*B*). We also tested the invasive capacity of the cell line through ECM barriers. We found that cells that have escaped the OIS pathway showed a dramatic increase in invasion compared with parental cells, toward both Matrigel and collagen I matrices (Fig. 8*C*). In addition, we also noticed that the steady-state level of villin, a marker of epithelial cell adhesion, was down-regulated in the emergent clones (Fig. 8*D*). Note, however, that this effect was already observed following RAS induction, suggesting that this effect on migration is an early characteristic of the RAS oncogene. These data suggested that emergent clones have acquired an enhanced ability to migrate, but because this also occurs during p21^{WAF1} up-regulation and senescence, this effect is probably not sufficient to allow tumor escape. This suggested to us that p21^{WAF1} and OIS inactivation might have also induced EMT in RasL cells. Using Western blotting and quantitative PCR analysis, we effectively observed that the expression of the E-cadherin was reduced in the emergent clones (Fig. 8*E*, *lanes 1–4*) whereas the vimentin mRNA was up-regulated (Fig. 8*F*, *lanes 1–4*). Interestingly, using FACS analysis, we also noticed an enhanced proportion of cells expressing the CD24^{low}/CD44^{high} phenotype during senescence escape (Fig. 8*G*). This confirms recent results obtained with mammary cells, showing that senescence escape and EMT induction generate cells with stem cells properties associated with the CD24^{low}/CD44^{high} phenotype (38). Note that a high expression of CD44 has been associated with an increased ability of HT29 cells to induce tumors in xenograft (39). Altogether, we concluded that OIS escape and p21^{WAF1} down-regulation are associated with cell dedifferentiation in HT29 cells.

Escape to the p21/OIS Pathway and EMT Induction Are Associated with Enhanced Survival and Bcl-xL/MCL1 Dependence—In response to p21^{WAF1} inactivation and the consequent genomic instability, cancer cells are expected to prevent cell death by the induction of compensatory antiapoptotic signals. We speculate that these survival signals are probably also necessary to allow the EMT transition. We thus analyzed whether acute or sustained RAS activation impact on survival signaling and focused our analysis on the Bcl-2 family of proteins (40). In parental cells, the up-regulation of RAS induced an increase in the expression of Bad and of the proapoptotic form of MCL1, namely MCL1-S (Fig. 9*A*). A decrease of Bim level was also noticed. In contrast, the expression of the Bcl-xL and MCL1 antiapoptotic proteins was not affected. Interestingly, these features were different in cells that have down-regulated the p21^{WAF1}/OIS suppressor pathway. In these cells, the expression level of MCL1-S was drastically decreased, and Bad was also down-regulated. In addition, the expression of the antiapoptotic protein Bcl-xL was higher compared with parental cells. As these expression profiles suggested an enhanced activity of Bcl-xL and MCL1, we investigated the role played by these two survival proteins during p21/OIS escape. To this end, Bcl-xL and/or MCL1 was depleted by siRNA, and apoptosis was evaluated by flow cytometry, using the anti-APO2.7 antibody (also known as 7A6) because its expression is restricted to dying, apoptotic cells. Importantly, results indicate that the codepletion of Bcl-xL and of MCL1, but not the depletion of either protein alone, led to significant apoptotic cell death rates in cells that have escaped the p21^{WAF1} suppressor pathway (Fig. 9*B*). In sharp contrast, parental HT29 cells and HT29 cells in which RAS had been activated for 48 h, remained essentially viable after Bcl-xL and MCL1 codepletion. These results indicate that cells that have escaped to the p21^{WAF1}-OIS protective pathway showed dependence on Bcl-xL/MCL1, probably as a response to one or more death signals specifically found in highly malignant cells expressing active RAS, having escaped OIS, resumed proliferation, and enhanced their invasion properties.

DISCUSSION

In this study, we have shown that the OIS program is conserved in the established HT29 colorectal cell line, in the absence of intact p53 and p16^{INK4} signaling. Following up-regulation of the RAS oncogene, p21^{WAF1} is up-regulated, and cell proliferation is arrested. This effect is not specific to HT29 cells or to H-RAS^{V12} because the same response has been detected in LS174T cells in response to B-RAF^{V600E}. Our results suggest that p14ARF and p53 are not involved in p21^{WAF1} up-regulation in HT29 cells and that, at least at the protein level, this effect relies on the p38 kinase as reported previously (30, 31). The cell cycle inhibitor prevents the expression of the *CDC25A* and *PLK1* genes, and we have observed that p21^{WAF1} binds to the PLK1 promoter to prevent its expression. Interestingly, we also noticed that some cells escaped this OIS pathway and that this progression is associated with a down-regulation of p21^{WAF1} expression. Senescence escape is associated with an increase in DNA damage, an induction of the EMT program, and an increase in the proportion of cells expressing the CD24^{low}/CD44^{high} phenotype. In response to this oncogenic stress, cells having escaped OIS rely on the survival pathway induced by Bcl-xL/MCL1 signaling.

FIGURE 5. **p21^{WAF1} binds to the PLK1 promoter to prevent its expression during OIS.** *A*, after incubation with 50 ng/ml doxycycline for 48 h, cells were stained with DAPI, and DNA cell content was analyzed by flow cytometry ($n = 3$). *B*, RNA was isolated following RAS induction, and the expression of the MYC, CDC25A, and PLK1 mRNAs was determined by quantitative real-time PCR. Data are quantified compared with the noninduced control and normalized to RPLPO expression ($n = 3 \pm$ S.D. (*error bars*)). *C*, cells were transfected with reporter gene as indicated together with control or p21^{WAF1}-expressing plasmids. After 2 days, cytoplasmic extracts were then prepared and processed to measure luciferase activity which was normalized compared with *Renilla* luciferase activity ($n = 3 \pm$ S.D.). *D*, HT29 cells were either transfected with p21-specific siRNA oligonucleotides or control oligonucleotides as indicated. The expression of the CDC25A and PLK1 mRNAs was analyzed by real-time PCR. The down-regulation of the cell cycle inhibitor was verified by Western blotting ($n = 3 \pm$ S.D.). *E*, soluble chromatin was prepared from cells and immunoprecipitated with antibodies directed against p21^{WAF1}, E2F1, or control anti-GAL4 antibodies. DNA was amplified using pairs of primers that cover the PLK1 proximal promoter. ChIP assays were quantified by real-time PCR compared with the GAL4 signal and control region ($n = 3 \pm$ S.D.).

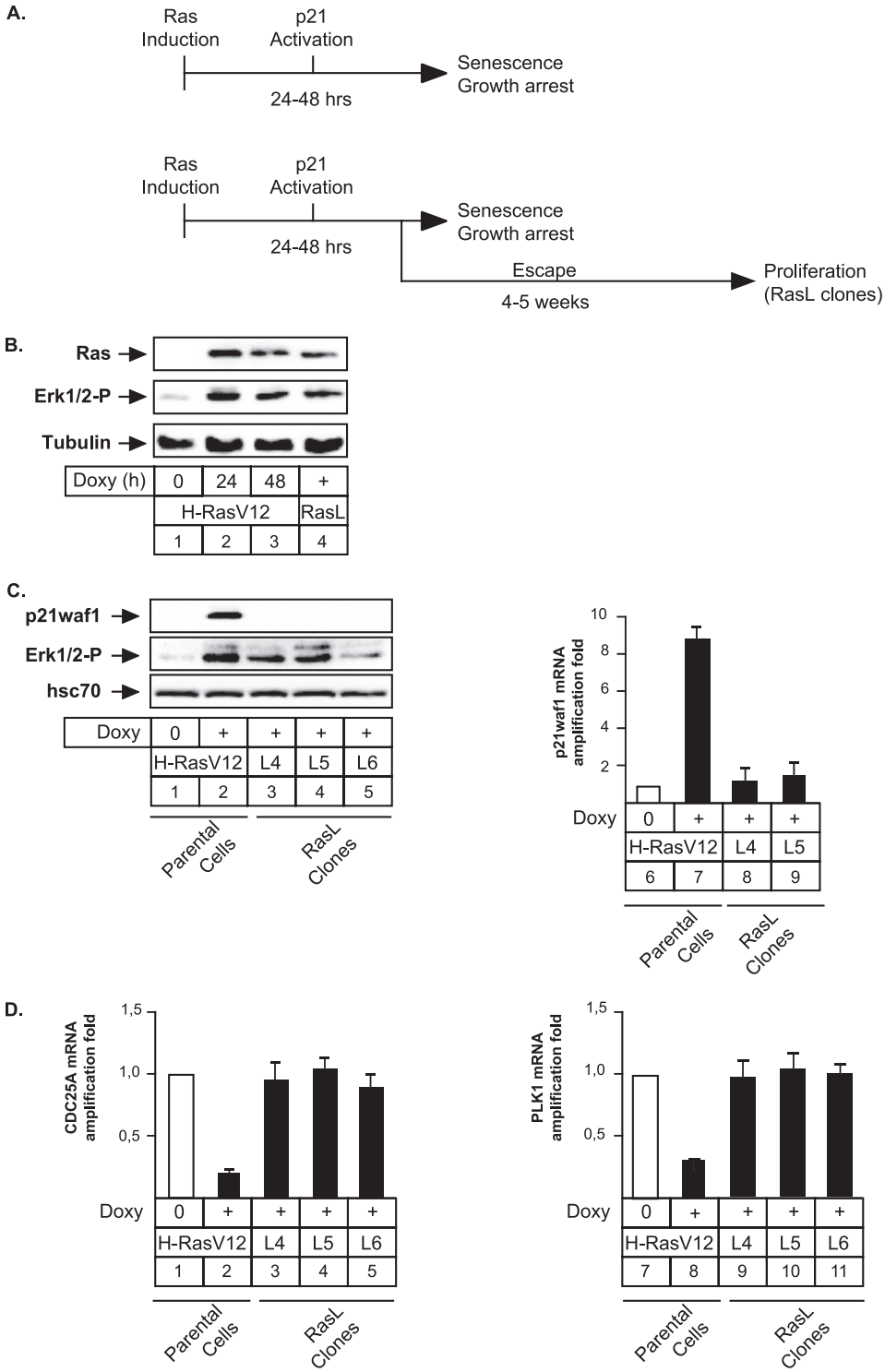


FIGURE 6. **OIS escape is associated with p21^{WAF1} down-regulation and CDC25A/PLK1 reexpression.** *A*, experimental procedure used to allow the growth of cells and the escape to RAS-mediated senescence (cells that escaped senescence were called RasL cells). *B*, Western blot analysis showing RAS and ERK1/2 expression in parental or RasL cells ($n = 3$). *C*, p21^{WAF1} expression analyzed in parental H29 cells or in different representative RasL clones by Western blot analysis (*lanes 1–5*, $n = 3$). In parallel, total RNA was isolated following RAS induction or in representative RasL cells, and the expression of the p21^{WAF1} mRNA was determined by quantitative real-time PCR (*lanes 6–9*, $n = 5 \pm$ S.D. (*error bars*)). *D*, expression of the CDC25A (*lanes 1–5*) and PLK1 (*lanes 7–11*) mRNAs following RAS induction or in RasL cells ($n = 3 \pm$ S.D.).

In response to abnormal oncogenic signaling, primary cells activate tumor suppressor pathways that rely on the initial activation of the ARF-p53 and p16^{INK4} proteins. In parallel, replication stress results in the early activation of DNA damage pathways that also function as tumor suppressors. In light of these observations, it was initially expected that cancer cells have to inactivate these tumor suppressor pathways to allow tumor progression. As expected, many established cell lines have inactivated p53 and INK4 signaling and, for this reason, it can be expected that OIS is essentially active in primary cells. At

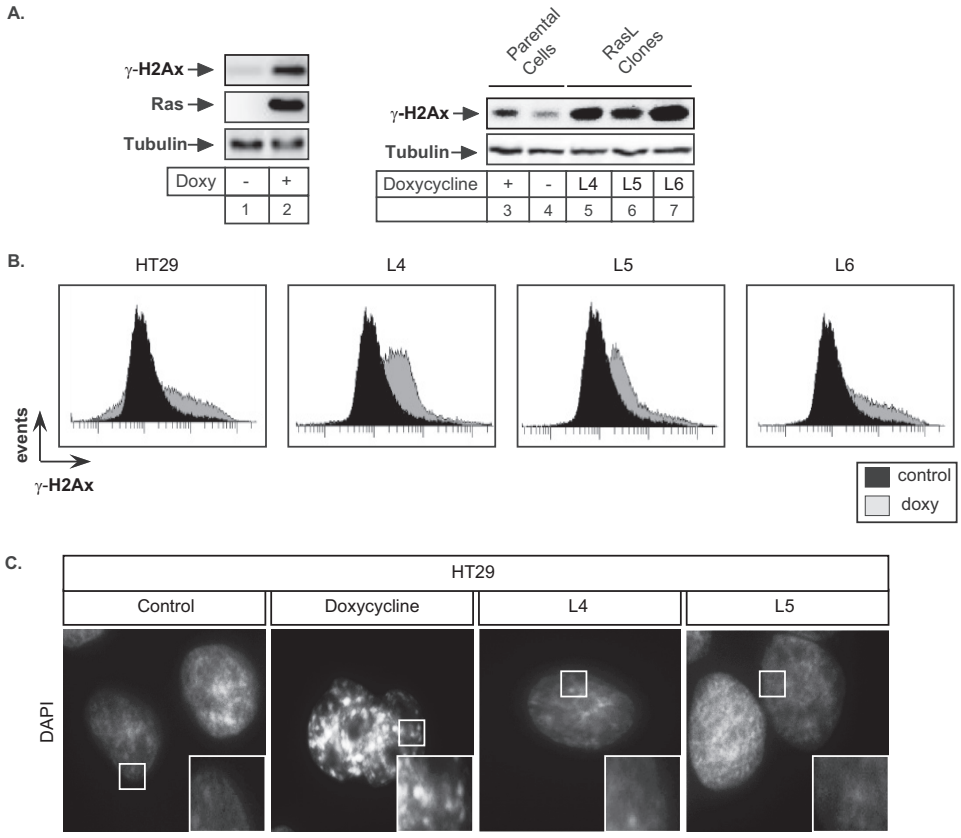


FIGURE 7. **DNA damage and SAHF down-regulation during p21^{WAF1}/OIS inactivation.** *A* and *B*, DNA double strand breaks were analyzed by Western blotting using polyclonal antibodies directed against the Ser^{139} phosphorylated form of histone H2Ax, following RAS induction in parental clones (48 h, *lanes 1–2* and *3–4*) or in representative RasL cells (*lanes 5–7*, $n = 3$) (*A*). The generation of DNA double strand breaks was also quantified by FACS analysis (*B*). *C*, SAHF formation and chromatin compaction were analyzed following RAS induction or in representative RasL cells by immunofluorescence using DAPI staining (one experiment representative of three is shown).

least in the cells used in this study, our results indicate that OIS is not only induced in primary cells but that this protective mechanism can still be active in some established colorectal cell lines despite the presence of acquired genetic abnormalities such as p53, and INK4 dysregulations. Recent results have already proposed this hypothesis, showing that the senescence program is conserved in established tumors and that oncogene inactivation is sufficient to reinduce cell cycle arrest (41). In addition, it has been shown recently that OIS can still be induced in established mammary cells (42). Our results indicate that p14^{ARF} and p53 are not involved in the response to the H-RAS^{V12} oncogene in HT29 cells, which is an important difference compared with primary cells. Although we were not able to detect the expression of p16^{INK4A}, the role of p15^{INK4B} remains to be established because recent results have reported the role of this protein during OIS induction (43).

Together, these results suggest that a complete inactivation of the OIS program is not necessary for initial cell transformation. It is well known that mutation in the APC tumor suppressor gene is one of the initial event of colon carcinogenesis, leading to constitutive β-catenin activation. However, other abnormalities are necessary to allow a complete cell transformation. For instance, RAS mutation is an important step in the activation of the β-catenin pathway and during carcinoma progression (44). In light of our results and others, we speculate that OIS is probably not completely inactivated following the initial β-catenin activation and that this suppressor pathway remains partially functional to restrain cell proliferation in response to secondary mutations. In addition, following senescence escape, our results also imply that apoptosis constitute a secondary fail-safe mechanism. In colorectal cells, we speculate that this suppressor pathway might become active when senescence is bypassed during the successive steps of cell transformation. Indeed, cells that have escaped the p21/OIS pathway harbor specific death signals that kill them once Bcl-xL and MCL1 are down-regulated by RNA interference. These pro-apoptotic signals and their relationship with RAS remain to be identified. Bcl-xL and MCL1 promote cell survival in great part by preventing some BH3-only proteins such as Bim or Puma to directly activate multidomain proteins and promote their cytotoxic activity. The loss of RAS-induced repression of Bim we found in escaped cells might thus contribute to these signals. Because p21^{WAF1} exerts antiapoptotic activities, it is also possible that its loss, which appears necessary to escape from senescence in our paradigm, contributes to enhance death signals in cells that have escape the OIS protective pathway.

We and others have previously described that p21^{WAF1} can function as a transcriptional inhibitor to prevent the expression of cell cycle genes (9–12, 14–18). This protein associates with several transcription factors such as NF-κB, STAT3, or E2F1 to bind to the promoters of genes involved in the activation of cell cycle progression such as *MYC*, *CDC25A*, or *WNT4*. We have

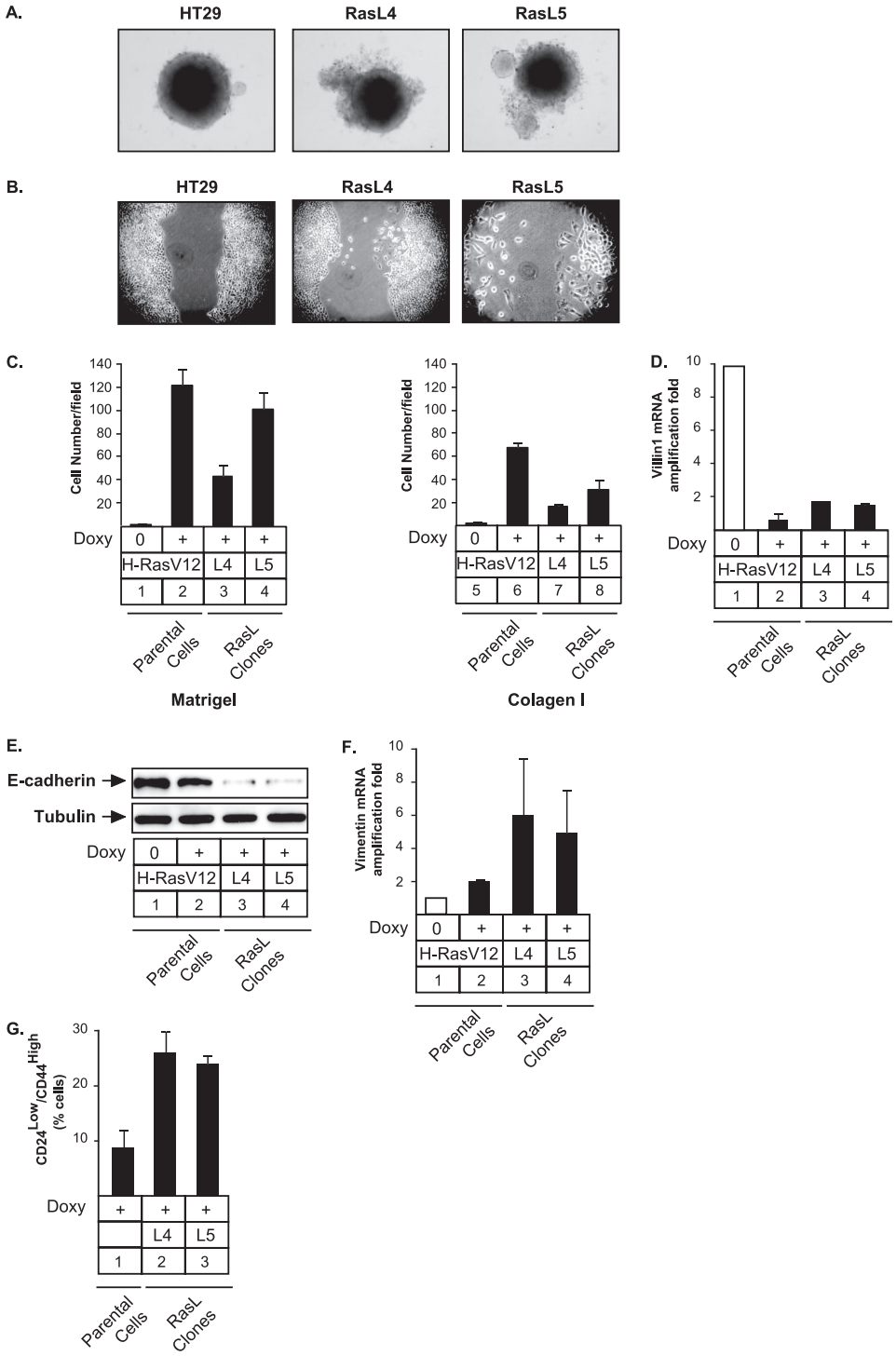


FIGURE 8. **p21^{WAF1}/OIS inactivation is associated with EMT induction.** *A*, using either parental or representative RasL cells, spheroids were allowed to grow for 8 days and visualized by microscopy (×200, $n = 3$). *B*, cell monolayers were wounded by removing cells with a standard 1-ml pipette tip. Following washing, cells were allowed to grow for 2 days ($n = 3$). *C*, transwell assay shows cell migration across Matrigel or collagen-precoated wells as indicated. Following loading, cells were incubated for 12 h at 37 °C, and cells that had migrated through the filter were counted and averaged from five randomly chosen microscopic fields using a ×20 objective. *D*, RNA was isolated following RAS induction or in representative RasL cells, and the expression of the villin mRNA was determined by quantitative real-time PCR and quantified compared with ribosomal protein large PO (RPLPO) expression ($n = 3 \pm$ S.D. (*error bars*)). *E* and *F*, following RAS induction or in representative RasL cells, E-cadherin expression was analyzed by Western blot analysis, using whole cell extracts. Tubulin expression was examined as a loading control (*E*, $n = 2$). In parallel, the expression of the vimentin (*lanes 5–8*) mRNAs was determined by quantitative real-time PCR. Data are quantified compared with RPLPO expression (*F*, $n = 3 \pm$ S.D.). *G*, percentage of cells expressing the CD24^{low}/CD44^{high} phenotype was determined by FACS analysis following RAS induction (*lane 1*) or in established RasL clones.

recently shown that p21^{WAF1} binds to these promoters in response to chemotherapy treatment to allow cell cycle arrest (13). However, to our knowledge, it has not been demonstrated previously that these transcriptional functions are also activated in response to an abnormal oncogenic activity in colorectal cancer cells. In addition, the association of p21^{WAF1} with

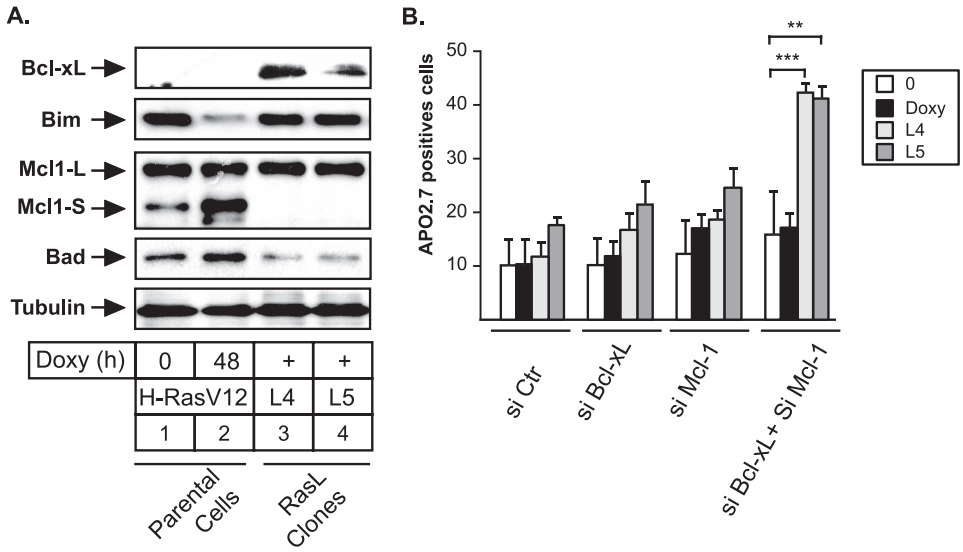


FIGURE 9. **Increased dependence onBcl-xL/MCL1 following p21^{WAF1} inactivation and OIS escape.** *A*, Western blot analysis of apoptotic and nonapoptotic regulators following RAS induction or in representative RasL clones ($n = 3$). *B*, HT29 cells transfected with either control or Bcl-xL and MCL1-specific siRNA oligonucleotides as indicated. Transfections were performed in parental cells treated or not with doxycycline or in two representative RasL clones. Apoptosis was evaluated by flow cytometry using APO2.7 staining ($n = 4 \pm$ S.D. (*error bars*)).

DNA has been characterized most of the time on genes activated at the G_0/G_1 transition of the cell cycle. Interestingly, using an isopropyl 1-thio-β-D-galactopyranoside-inducible vector (27), it has already been suggested that the overexpression of p21^{WAF1} leads to the down-regulation of G_2-specific genes to prevent progression toward mitosis. In this study, we further confirmed this observation, showing that p21^{WAF1} binds to the *PLK1* promoter to prevent its expression in response to the RAS oncogene. As reported previously, we speculate that this effect is due to the inactivation of transcriptional regulators such as CBP and to histone deacetylation. As stated above, OIS is associated with chromatin reorganization into senescence-associated heterochromatin foci. Silencing depends on the Rb pathway and is associated with enhanced histone H3 trimethylation and recruitment of the HP1 protein on proliferative genes. Therefore, it will be interesting to determine whether the binding of p21^{WAF1} to the *PLK1* promoter is associated with the recruitment of transcriptional repressors such as Rb or H3K9me3 methyltransferases. As expected, PLK1 expression was reactivated following p21^{WAF1} down-regulation and OIS escape. Interestingly, we have also noticed that cells that escape this suppressor mechanism proliferate despite significant DNA damage. Theoretically, in the absence of intact p53-p21 signaling, inactivation of CDC25C or spindle checkpoint activation is expected to prevent the propagation of damaged DNA (45, 46). In addition, we and other have proposed that the Aurora-A-PLK1 pathway plays an important role to prevent G_2 progression during DNA damage (23, 47, 48). Therefore, we speculate that these G_2/M checkpoints are also dysregulated during OIS escape. Because cells expressing the RAS oncogene have been recently reported to be addicted to the PLK1 kinase (49), it will be interesting to determine whether the Aurora-A-PLK1 pathway is involved in OIS escape in our experimental conditions.

Besides regulating cell cycle genes, it has also been shown recently in breast cancer cell lines that p21^{WAF1} inhibits the epithelial mesenchymal transition in response to the RAS oncogene (50). The cell cycle inhibitor can prevent the repression of the E-cadherin gene by the Twist transcription factor. In addition, these results also indicate that p21^{WAF1} reduces the proportion of cells expressing the CD24^{low}/CD44^{high} phenotype in response to oncogenic transformation. Finally, some of the genes that are overexpressed in embryonic stem cells are also repressed by the cell cycle inhibitor. In light of these results, we speculate that p21^{WAF1} also prevents EMT in colorectal cancer and that this effect might be due to a direct binding of the cell cycle inhibitor to the corresponding promoters. The inactivation of this protein during OIS escape would restore the expression of these genes and allow EMT. Importantly, dedifferentiation of transformed epithelial cells and loss of E-cadherin are associated with chemotherapy resistance (51). It is striking to note that both p21^{WAF1} inactivation and Bcl-xL/MCL1 up-regulation occur at the same time in our experimental conditions. The dysregulation of these two pathways is a key event that can also lead to chemotherapy resistance. Unraveling the mechanisms that lead to the combined loss of p21^{WAF1}, of MCL1-S, and the enhanced Bcl-xL expression during OIS escape is thus of particular relevance, as these events probably play a critical role in maintaining the survival of transformed epithelial cells with a highly malignant phenotype.

Acknowledgments—We thank Dr. T. Uchiumi and Dr. A. Schulze for providing PLK reporter and Raf constructs.

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